PTHrP Is Endogenous Relaxant for Spontaneous Smooth Muscle Contraction in Urinary Bladder of Female Rat

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Acute bladder distension causes various morphologic and functional changes, in part through altered gene expression. We aimed to investigate the physiologic role of PTHrP, which is up-regulated in an acute bladder distension model in female rats. In the control Empty group, bladders were kept empty for 6 hours, and in the Distension group, bladders were kept distended for 3 hours after an artificial storing-voiding cycle for 3 hours. In the Distention group bladder, up-regulation of transcripts was noted for 3 genes reported to be up-regulated by stretch in the cultured bladder smooth muscle cells in vitro. Further transcriptome analysis by microarray identified PTHrP as the 22nd highest gene up-regulated in Distension group bladder, among more than 27 000 genes. Localization of PTHrP and its functional receptor, PTH/PTHrP receptor 1 (PTH1R), were analyzed in the untreated rat bladders and cultured bladder cells using real-time RT-PCR and immunoblotting, which revealed that PTH1R and PTHrP were more predominantly expressed in smooth muscle than in urothelium. Exogenous PTHrP peptide (1-34) increased intracellular cAMP level in cultured bladder smooth muscle cells. In organ bath study using bladder strips, the PTHrP peptide caused a marked reduction in the amplitude of spontaneous contraction but caused only modest suppression for carbachol-induced contraction. In in vivo functional study by cystometrogram, the PTHrP peptide decreased voiding pressure and increased bladder compliance. Thus, PTHrP is a potent endogenous relaxant of bladder contraction, and autocrine or paracrine mechanism of the PTHrP-PTH1R axis is a physiologically relevant pathway functioning in the bladder. (***Endocrinology*** **154: 2058–2068, 2013)**

Bladder distension caused by bladder outlet obstruction is a condition frequently encountered in urologic clinics that causes various morphologic and functional changes to the bladder, including matrix remodeling, muscular hypertrophy, bladder decompensation, and bladder overactivity (1–4). According to the myogenic bladder remodeling hypothesis, transcriptional changes in gene expression are involved in such changes (5), and we have previously shown that collagen I/III and connexin 43 are genes that affect myogenic changes after bladder outlet obstruction (1, 6).

Adam and colleagues (7) performed comprehensive transcriptome analysis on cultured bladder smooth muscle cells (BSMCs) subjected to cyclic stretch to study the effect of bladder distension on gene expression in vitro. Transcriptional changes found in the in vitro model were subsequently validated using an ex vivo bladder distension model. Several genes, including heparin-binding epidermal growth factor (EGF)-like growth factor, cyclooxygenase2, and thrombomodulin, were found to be up-regulated in both the in vitro and ex vivo studies, indicating that genetic changes in the distended bladder are caused, at least in part, by mechanical

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Abbreviations: AC, adenylyl cyclase; BSMC, bladder smooth muscle cell; Cch, carbachol; EGF, epidermal growth factor; EIA, enzyme immunoassay; PKA, protein kinase A; PTH1R, PTH/PTHrP receptor 1; α-SMA, α-smooth muscle actin; SQ22536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine; UC, urothelial cells.

stretch to smooth muscle cells. However, such comprehensive transcriptional analysis has not yet been reported for in vivo acute bladder distension, leaving the physiologic relevance of the findings uncertain.

PTHrP was originally identified as a cause for hypercalcemia in paraneoplastic syndrome. Both PTH and PTHrP act as ligands for PTH/PTHrP receptor 1 (PTH1R). PTH is expressed solely in the parathyroid, whereas PTHrP is expressed in most tissues in the body. Whereas PTH functions as an endocrine hormone with bone and kidneys as the main target organs, PTHrP is considered to be an autocrine or paracrine ligand for PTH1R in various organs with diverse functions such as bone growth regulation, calcium transport, mammary gland development, tooth eruption, and smooth muscle relaxation (8-10). There are two types of PTH receptor, PTH1R and PTH2R, but PTH2R is known to be activated solely by PTH and not by PTHrP (11). Thus, PTH1R is considered to be the only functional target of PTHrP in these organs. PTHrP was found to be up-regulated by bladder distension and stretch of smooth muscle cells and was identified as a relaxant of bladder smooth muscle in 2 early reports (12, 13). However, its effect on carbachol (CCh)-induced muscle contraction. as shown in these initial reports, was only modest, and no further functional studies have been undertaken since. In the initial studies, the expression and localization of PTH1R in the bladder, the downstream pathway of PTH1R, and the in vivo effect of PTHrP in the bladder were not investigated (13). In short, the study on PTHrP as a functional molecule in the bladder has been interrupted, except for a short report on its immunohistochemical localization without functional analysis (14).

In the present report, PTHrP is rediscovered as a highly up-regulated molecule in a rat bladder acute distension model in vivo, using microarray analysis. Thus, we subsequently reinvestigate the physiologic role of PTHrP in the bladder as a candidate gene for functional changes after acute bladder distension and demonstrate a relevant physiologic effect of PTHrP on spontaneous contraction of bladder smooth muscle (detrusor muscle) and in vivo bladder function.

Materials and Methods

Animals

Eight-week-old female Sprague Dawley rats (180–220 g, SLC Japan, Hamamatsu, Japan) were used for all experiments in this study. Rats were housed at constant room temperature with a 14-hour light (7:00 AM to 9:00 PM), 10-hour dark (9:00 PM to 7:00 AM) cycle. Food and water were available ad libitum. Rats were treated according to guidelines (15). All animal experiments were approved by the Kyoto University and Nagoya City University animal experiment committees, and all animals used in this study were treated according to The Guidelines for Animal Experimentation of the Experimental Animal Center of Kyoto University and Nagoya City University

Cell culture

BSMCs and urothelial cells (UCs) were isolated from rat bladders using a previously described procedure (1, 16, 17). After 2 passages, BSMCs were seeded at 10^5/2 mL in 6-well plates for RNA and protein extraction and at 10^4/100 μL in 96-well plates for cAMP-enzyme immunoassay (EIA) assays.

Rat bladder distension model

For the in vivo bladder distension model, rats were anesthetized by sc urethane injection (1.2 g/kg). Saline was infused into the urinary bladder through a transurethral catheter, and the intravesical content was drained through the urethra around the transurethral catheter. Rats were randomly divided into 3 groups: in the Empty group, bladders were kept empty for 6 hours; in the Storing-Voiding cycling group, bladders were kept on a storing and voiding cycle with continuous saline infusion (1 mL/h) for 3 hours; in the Distension group, bladders were kept on a storing-voiding cycle as in the Storing-Voiding cycling group, after which the bladders were kept distended by clamping the urethra at the volume just before voiding for an additional 3 hours (n = 4–5 per group, Figure 1A). After the experiment described, rats were humanely destroyed by with cervical dislocation, and whole bladder tissues were used for extraction of RNA.

Because 3-hour distension was expected to be too short for protein expression, we used gradual distension model for protein retrieval, with a method described previously (12), and distension time was extended to 6 hours. Six hours Empty group and 6 hours Distension group were compared (n = 2 for representative immunoblot, and n = 6 for densitometry), and bilateral ureteral ligation was performed in the Empty group, because urine drainage from the urethra outside the transurethral catheter was not reliable to keep the bladder empty for an additional 3 hours. Sham operations (laparotomy and isolation of bilateral ureters) were performed in the Distension group. Before closing the abdominal wall, 5 mL saline was infused ip in each group. Retrieval of the bladder was done in the same manner as described above.

Tissue separation for localization analyses

For localization analyses, untreated rat bladders were separated to muscle layer and urothelium using ophthalmology scissors. Each tissue has undergone RNA or protein extraction.

Real-time RT-PCR

Total RNA was extracted from bladder tissue or cells and treated with DNase I using RNeasy Mini kits (QIAGEN, Hilden, Germany) and RNase-Free DNase Sets (Qiagen), according to the manufacturer's protocols. cDNA was synthesized from total RNA using First-Strand cDNA Synthesis Kits (GE Healthcare, Waukesha, Wisconsin). Real-time PCR was performed using SYBR green PCR Master Mix (Life Technologies, Carlsbad, California) and recorded by 7300 Realtime PCR system (Life Technologies) (n = 6). The thermal cycling conditions were 94°C for 15 s, 57°C for 15 s, and 72°C for 1 min. Values were adjusted by the expression levels of 18s rRNA. Primer sequences are shown in Table 1.

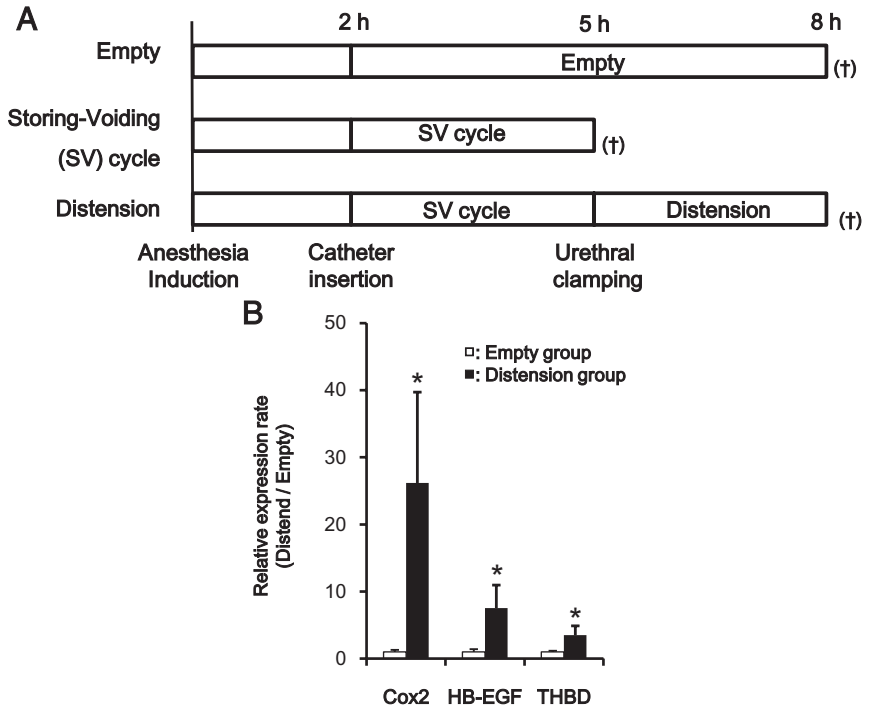

Figure 1. An in Vivo Acute Bladder Distention Model A, Schematic of the rat bladder acute distension model. †, time of sacrifice; SV, storing-voiding. B, Characterization of the rat bladder distention model. The distended rat bladder showed transcriptional changes similar to bladder smooth muscle cells subjected to cyclic stretch, as previously reported. The value of the empty group is set at 1 (n = 6, $P < .05$). Cox2, cyclooxygenase 2; HB-EGF, heparin-binding EGF-like growth factor; THBD, thrombomodulin.

Affymetrix GeneChip array

Comprehensive gene expression analysis was performed for total RNA isolated from the Empty and Distended rat bladders (n = 3 each) using the Affymetrix GeneChip Rat Gene 1.0 ST Array containing probes for more than 27 000 well-annotated genes. RNA concentration and purity were determined by measurement of A260/A280 ratios with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California). Aliquots of 100 ng total RNA were used for each sample. Target labeling, array hybridization, washing, and staining were performed as described in the GeneChip Whole Transcript Sense Target Labeling manual (http://www.affymetrix.com). Arrays were scanned using the GeneChip Scanner 3000 7G (Affymetrix).

Data acquisition was performed with the GeneChip Command Console Software (AGCC). Data analysis was performed with GeneSpring GX Version 11.5.1 (Agilent Technologies) under the following conditions: summarization algorithm, RMA16; confidence level, all; baseline options, do not perform baseline transformation. In a total of 27 342 genes on the microarray, genes with more than 80% signal intensity (22 726 genes) were used for comprehensive analysis. Expression changes between the Distension group and the Empty group were calculated.

Immunoblotting

After washing with ice-cold PBS, rat bladder tissue or cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM sodium chloride, 2 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 50 mM sodium fluoride, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride supplemented with protease cocktail inhibitors (Complete Mini; Roche, Mannheim, Germany) for PTHrP, and in radioimmune precipitation assay buffer with protease inhibitor (Nacalai Tesque, Kyoto, Japan) for PTH1R. The extracts were centrifuged at 13 000 × *g* at 4°C for 20 minutes. Total cellular protein concentrations were determined using a protein assay reagent (Bio-Rad Laboratories, Inc., Richmond, California). Protein lysates (for PTHrP: 80 μg, for PTH1R: 20 μg) were subjected to SDS-PAGE using 15% gel for PTHrP, and 8% gel for PTH1R, and transferred to polyvinylidene difluoride membranes (Millipore Corp, Bedford, Massachsetts). Membranes were immunoblotted with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and developed for reading by enhanced chemiluminescence (Pierce Western Blotting Substrate Plus; Thermo Scientific, Rockford, Illinois). Images were acquired with the LAS-4000 imaging system (Fujifilm Life Science, Tokyo, Japan) (18, 19). Anti-PTHrP (PC-09, Merck KGaA, Darmstadt, Germany; diluted at 1:100), anti-PTH1R (PRB-630P; Covance Laboratories, Madison, Wisconsin; diluted at 1:1000), anti-α-smooth muscle actin (SMA) (ab5694, Abcam, Cambridge, UK; diluted at 1:10 000), anti-cytokeratin 18 (C8541; Sigma Aldrich, St Louis, Missouri; diluted at 1:4000), and anti-β-actin (AC-15; ab6276 Abcam; diluted at 1:25000) were used as primary antibodies.

cAMP-EIA assay

BSMCs were seeded at 1.0×10^4 per well in 96-well plates and incubated for 24 hours in DMEM (Invitrogen, Carlsbad, California) supplemented with 10% fetal calf serum. The medium was changed to serum-free DMEM 24 hours before the assay. One hour before the assay, 10 nM PTHrP(1-34) amide (4502-v, Peptide Institute, Osaka, Japan), an agonist of PTH1R, or vehicle was added with 3-isobutyl-1-methylxanthine (I5879; Sigma Aldrich; final concentration 0.1 mM). In addition, either 10 nM PTHrP(7-34) amide (4512-v; Peptide Institute), an antagonist of PTHrP, or 10 mM 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536) was added for PTHrP treatment. Intracellular cAMP concentrations were assayed using cAMP-EIA kit (RPN225; GE HealthCare) (n = 6) (20).

Organ bath study

Tissue preparations

A total of 30 rats without any treatment were anesthetized with isoflurane inhalation and exsanguinated by decapitation. The bladders were removed and pinned down in a dissecting dish

Table 1. Real-time RT-PCR Primer Information.

Gene		Primer Sequence (5′→3′)
18s rRNA	F	actcaacacgggaaacctca
	R	aaccagacaaatcgctccac
Cox2	F	accagcaggctcatactgatagg
	R	ctgggtcgaacttgagttgga
HB-EGF	F	ctgagatggcggttccttaca
	R	ggcccagtcagggtagcaa
THBD	F	gtttcaaatgtgcagaggatcct
	R	tggcccaatatgtctgaagatg
PTHrP	F	cagccgaaatcagagctacc
	R	ctcctgttctctgcgtttcc
PTH1R	F	gcggacgatgtctttaccaaa
	R	ttggctgctgtgtgcagaa
aSMA	F	gctgtgctatgtcgctctgg
	R	aatgaaagatggctggaagagg
Calponin	F	acaggccgccgcatcgggagcaac
	R	cagcctggctggcgcccttgttggta
SM-MHC	F	catgctacaagatcgtgaagaccagtcc
	R	ccaaagcgggaggagttgtcattcttg
CK20	F	atgcggataactgtggaagc
	R	cctccacgttgacattgttg
UP3a	F	ctgacccttgtggtgactt
	R	gatgggatccgaccatagtg

Cox2, cyclooxygenase 2; CK20, cytokeratin 20; F, forward, R, reverse; HB-EGF, heparin-binding EGF-like growth factor; SM-MHC, smooth muscle myosin heavy chain; THBD, thrombomodulin; UP3a, uroplakin 3a.

with the urothelial side facing up. The urothelial layer was dissected away from the smooth muscle layer using ophthalmology scissors. After the sample was turned upside down, the outer smooth muscle layer was removed, leaving the inner smooth muscle layer for use in the study.

Isometric tension recordings

Silk threads were tied around both ends of the smooth muscle strips (~8–10 mm long; 1–3 mm wide). Preparations were transferred to 2 mL organ baths and superfused with warmed (35°C) physiologic salt solution (21) at a constant flow rate (2-3 mL/min). One thread was fixed to the bottom of the organ bath, while the other was connected to an isometric force transducer connected to a bridge amplifier. Isometric tension changes were digitized using a Digidata 1200 interface (Axon Instruments, Inc, Foster City, California) and stored on a personal computer for later analysis. After 30 minutes of incubation with warmed physiologic salt solution, preparations were stretched appropriately to exhibit spontaneous phasic contraction. Preparations were then left to equilibrate for about 30–60 minutes until spontaneous phasic contraction became stable in both amplitude and frequency.

Cystometrogram

For cystometrogram, a total of 32 rats without any treatment were anesthetized with an sc injection of urethane (0.9 g/kg). After laparotomy, the bladder was exposed and both ureters were tied and cut. A polyethylene catheter (PE60; Clay-Adams, Parsippany, New Jersey) with a needle was inserted into the bladder dome and held in place with a purse-string suture. The catheter was connected via a 3-way stopcock to a pressure transducer (DX-360; Nihon Kohden, Tokyo, Japan) for recording of the intravesical pressure, and to a syringe pump (STC-521; Terumo Corp., Tokyo, Japan) for infusion of saline into the bladder. After checking for leaks, the bladder was emptied by gently pushing the hypogastrium. In the same session, a needle was inserted into one of the femoral veins for iv injection. Cystometrogram was carried out by filling the bladder with saline at a rate of 6 mL/h to elicit repetitive voiding. Intravesical pressure signals were conveyed to a carrier amplifier (AP-621G; Nihon Kohden) and continuously recorded on a pen recorder (RJG-4124; Nihon Kohden). After intercontraction interval and amplitudes of bladder contractions had stabilized, 10 μg PTHrP(1-34) amide in 180 μL saline or saline only was administered iv. Ten sequential micturition cycles were selected immediately before and after drug administration. Average intercontraction intervals and pressure of bladder before and after the filling phase and at the peak of contraction were measured to calculate maximum intravesical pressure and compliance during filling phase. The percentages of change before and after drug administration were calculated (22, 23).

Statistical analysis

Data are expressed as mean ± SEM. Microsoft Excel was used for statistical analysis. Student's t-tests and paired t-tests were performed when appropriate. $P < .05$ was accepted as significant.

Agents

PTHrP(1-34)amide (4205-v) and PTHrP(7-34)amide (4215-v) were obtained from Peptide Institute (Osaka, Japan). Cch, forskolin, 8-bromo-cAMP, and SQ22536(S-153) were obtained from Sigma-Aldrich (St Louis, Missouri). H-89 was obtained from Nacalai Tesque (Kyoto, Japan).

Results

Creation and characterization of the model

To investigate transcriptional changes during acute bladder distension, we used a rat bladder acute distension model (Figure 1A). To compare this in vivo model with BSMCs under cyclic stretch in vitro, we investigated the expression levels of 3 genes reported to be up-regulated in stretched BSMCs: cyclooxygenase 2, heparin-binding-EGF, and thrombomodulin (7). Real-time RT-PCR showed that all 3 selected genes were also significantly up-regulated after bladder distension in our in vivo model (Figure 1B), demonstrating that genetic changes in this in vivo model reflect the effects of stretch on smooth muscle.

Affymetrix GeneChip Array

We next performed comprehensive transcriptome analysis of the animal model. In a total of 27 342 genes on microarray, 353 genes were significantly up-regulated more than 2-fold by bladder distension, and 198 genes were down-regulated more than 2-fold. The 30 genes with highest level of up-regulation are shown in Table 2. Following literature study of these genes, we focused on

Table 2. Result of Affymetrix Genechip array.

Fold Change	Gene Description	Gene Symbol	*P* Value
20.827	Activating transcription factor 3	Atf3	.023
18.166	Activity-regulated cytoskeleton-associated protein	Arc	.002
16.859	IL-6	Il6	.049
12.006	Chemokine (C-X-C motif) ligand 2	Cxcl2	.021
10.632	Hyaluronan synthase 1	Has1	.023
10.556	TNF receptor superfamily, member 12a	Tnfrsf12a	.001
9.701	Cysteine-rich, angiogenic inducer, 61	Cyr61	$7 \times 10–5$
9.282	Serine (or cysteine) peptidase inhibitor, clade E, member 1	Serpine1	.012
8.859	OTU domain containing 1	Otud1	.026
8.686	Neuronal pentraxin 2	Nptx2	.0006
8.599	Regulator of calcineurin 1	Rcan1	.006
8.526	Xin actin-binding repeat containing 1	Xirp1	.045
8.226	Nuclear receptor subfamily 4, group A, member 1	Nr4a1	.006
7.866	Apolipoprotein L domain containing 1	Apold1	.009
7.815	Connective tissue growth factor	Ctgf	.003
7.683	CCR4 carbon catabolite repression 4-like (*S. cerevisiae*)	Ccrn4l	.035
7.251	Suppressor of cytokine signaling 3	Socs3	.047
7.057	Glutamine-fructose-6-phosphate transaminase 2	Gfpt2	.014
6.874	FBJ osteosarcoma oncogene	Fos	.001
6.645	Tribbles homolog 1 (*Drosophila*)	Trib1	.012
6.582	Fos-like antigen 1	Fosl1	.038
6.167	**PTH-like hormone (= PTHrP)**	**Pthlh (=Pthrp)**	**.002**
5.869	Epiregulin	Ereg	.075
5.834	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*); translocated to, 11	Mllt11	0.009
5.733	Similar to RIKEN cDNA 6330406I15	RGD1307396	.024
5.713	BTG family, member 2	Btg2	.006
5.567	Major facilitator superfamily domain containing 2	Mfsd2	.002
5.476	Nucleoporin 62 C-terminal like	Nup62cl	.001
5.435	Similar to 1300014I06Rik protein	RGD1311307	.012
5.337	Poliovirus receptor	PVR	.002

The table is a list of top 30 genes highly up-regulated in distended bladder. Bold, 22nd highest up-regulation.

PTHrP (bold in Table 2, 22nd highest up-regulation; fold change = 6.17; $P = .002$) as a candidate of functional gene associated with acute distension, because of incomplete characterization done thus far for this molecule in the bladder.

Expression study of PTH/PTH1R in the bladder

Up-regulation of PTHrP transcription after acute bladder distension suggested by Affymetrix GeneChip Array was validated by real-time RT-PCR, with a 4.97 ± 0.75-fold up-regulation in the Distension group compared with the Empty group ($P = .001$, Figure 2A). The increase in PTHrP protein level was demonstrated by immunoblotting of the tissues from the 6-hour Empty group and the 6-hour Distension group, and densitometry showed a significant difference in PTHrP expression between the two groups ($P = .011$, Figure 2B-1,2).

As the next step, the localization of PTHrP was investigated in bladder smooth muscle and urothelium. The accuracy of separating the urothelium from the muscle was validated by examining mRNA levels of 3 smooth muscle markers (α-SMA, smooth muscle myosin heavy chain, and calponin) and 2 urothelial markers (uroplakin 3a and cytokeratin 20), all of which showed distinct tissue-specific localization. A significantly higher level of PTHrP mRNA was expressed in smooth muscle tissue than in urothelium (6.07 ± 2.56-fold compared with urothelium; $P = .046$, Figure 2C). For assessing the putative target of PTHrP in the bladder, we also examined the localization of PTH1R, which is the only type of PTH/PTHrP receptor targeted by PTHrP (11). In contrast with the dynamic changes in PTHrP levels, the expression of the PTH1R transcript remained relatively stable under bladder distension (Figure 2A). PTH1R was predominantly expressed in smooth muscle both in mRNA level (4.43 ± 0.28-fold compared with urothelium; $P = 1.05 \times 10^{-5}$, Figure 2C) and in protein level (Figure 2D), suggesting that PTHrP is likely to function on smooth muscle rather than on urothelium.

Effect of PTHrP(1-34) treatment on intracellular cAMP of BSMCs

Because PTH1R is predominantly localized in the muscle layer, we focused on the physiologic effects of PTHrP

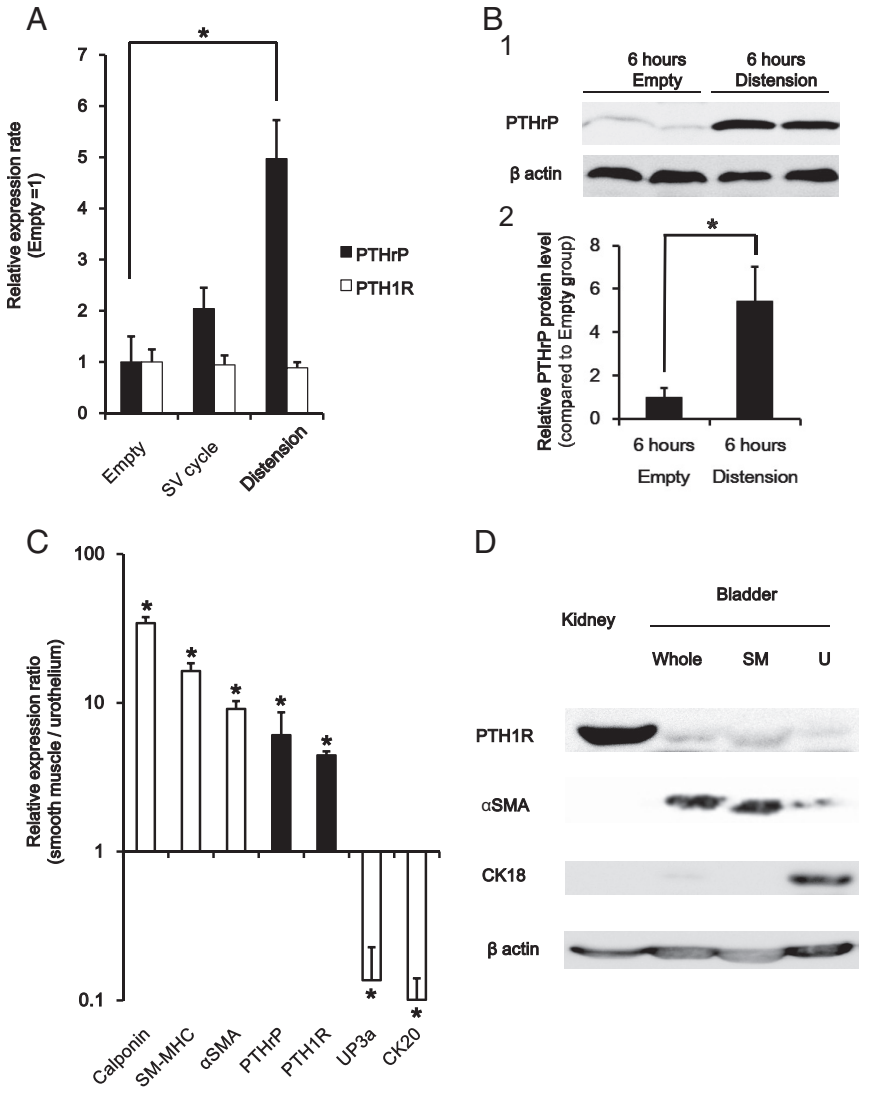

Figure 2. Expression Profiles of PTHrP and PTH1R A, Up-regulation of PTHrP mRNA by bladder distention was certified by real-time RT-PCR. The value of the empty group is set at 1 (n = 4-5; $*P < .05$). On the contrary, PTH1R mRNA did not change significantly with bladder distention. SV, storing-voiding. B, PTHrP protein was up-regulated in the distended bladder. 1. Representative immunoblot of 6 hours Empty group and 6 hours Distension group (n = 2). 2. Densitometry of immunoblot result from another independent experiment. Relative PTHrP protein level was calculated from the PTHrP signal density adjusted by the signal density of β-actin (n = 6; $P = .011$). $*P < .05$. C, Real-time RT-PCR showing localization of PTHrP and PTH1R mRNA in the bladder (n = 5). Expression levels of 3 smooth muscle markers (Calponin, smooth muscle myosin heavy chain [SM-MHC], and α-SMA) and 2 urothelial markers (uroplakin 3a [UP3a] and cytokeratin 20 [CK20]) are shown as open bars. PTHrP and PTH1R protein mRNA expression were both dominant in the smooth muscle layer (n = 5; $P = .046$ and 1.0×10^{-5}, respectively; $P < .05$). D, Immunoblot showing that the PTH1R protein is predominantly expressed in smooth muscle of the bladder. Separation of the smooth muscle layer and the urothelial layer was validated by immunoblotting with α-SMA and cytokeratin 18 antibodies. SM, smooth muscle layer; U, urothelial layer.

peptide on bladder smooth muscle. In parallel with the findings from the separated bladder tissues, BSMCs isolated from rat bladders expressed significantly more PTH1R than UC, both in mRNA level (Figure 3A-1), and in protein level (Figure 3A-2). In smooth muscle of other organs, PTHrP is known to act as a smooth muscle relaxant via the adenylyl cyclase (AC)-cAMP-protein kinase A (PKA) pathway (24–26). Therefore, we first investigated the effect of PTHrP on the intracellular cAMP concentration of BSMCs. As expected, 10 nM PTHrP(1–34) treatment increased the intracellular cAMP concentration, and this effect was significantly inhibited by 10 nM PTHrP(7–34), a competitive antagonist of PTHrP, and 10 μM SQ22536, an adenylyl cyclase (AC) inhibitor (Figure 3B), confirming that PTHrP increased intracellular cAMP levels via PTH1R and AC.

Effect of PTHrP on spontaneous contraction of bladder smooth muscle

Although PTHrP was previously reported to be a bladder muscle relaxant, its effect was only modest when evaluated against Cch-induced contraction (12, 13). Considering the relevant relaxant effect of PTHrP on other types of smooth muscle (24), we hypothesized that the bladder PTHrP/PTH1R axis may preferentially suppress spontaneous contraction not tested in previous reports. Thus, we examined the effect of PTHrP on spontaneous contraction in rat bladder strips in organ bath study.

Indeed, 10 nM PTHrP(1–34) treatment potently suppressed the spontaneous contraction amplitude of the bladder detrusor muscle in a dose-dependent manner (Figure 4, A and C), but did not change the area under the curve (Figure 4D) and the intercontraction interval (Figure 4E). Compared to the effect on amplitude, the baseline decrease after PTHrP(1–34) treatment was relatively mild (Figure 4B).

PTHrP acts via the AC-cAMP-PKA pathway in smooth muscle of many organs including blood vessels, gall bladder, and trachea (10, 24–26). Indeed, 8-bromo-cAMP, a cAMP analog, and forskolin, an AC activator, have inhibitory effect similar to that of PTHrP (Supplemental Figure 1, A–C, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Further, we attempted

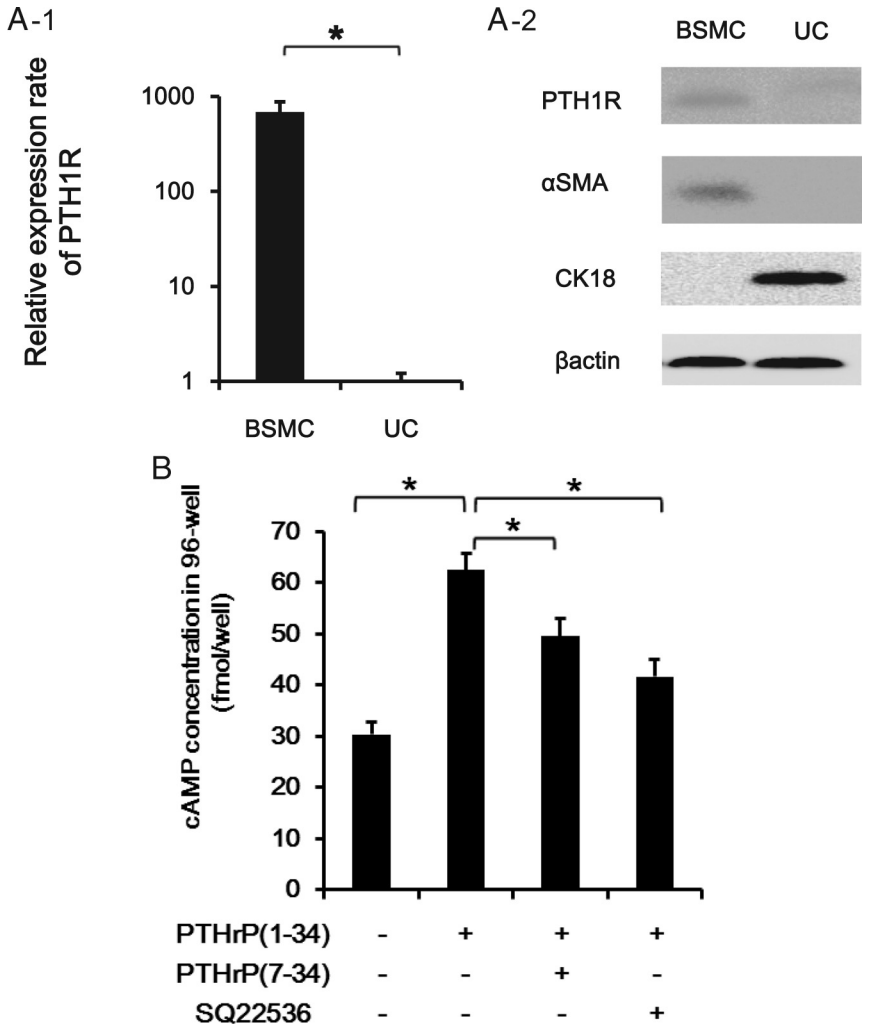

Figure 3. PTHrP and PTH1R Pathway Increases Intracellular cAMP through the AC-cAMP-PKA Pathway A, BSMCs expressing PTH1R. A-1, Real-time RT-PCR showing that PTH1R expression is more than 100-fold higher in BSMCs than in UCs in mRNA level. *$P < .05$ against UC expression set as 1. A-2, Immunoblot showing higher PTH1R expression in BSMCs than in UCs. B, Intracellular cAMP increased by treatment with 10 nM PTHrP(1–34), which was inhibited by the addition of 10 nM PTHrP(7-34), the antagonist peptide, and 10 μM SQ22536, the adenyl cyclase inhibitor. *$P < .05$, against the cells treated with PTHrP(1–34) alone. CK18, cytokeratin 18.

to explore the downstream pathway of PTH1R by pharmacologic inhibition using the protein kinase A inhibitor H89 (10 μM) (27), or the AC inhibitor SQ22536 (300 μM) (28). However, those inhibitors themselves suppressed spontaneous contraction (Supplemental Figure 2A for H89; Supplemental Figure 2B for SQ22536), and thus further inhibition study was not possible.

Effect of PTHrP on Cch-induced contraction of bladder smooth muscle

The effects of PTHrP on Cch-induced contraction of bladder smooth muscle were examined (12, 13). Consistent with the inhibitory actions of PTHrP reported in previous studies, PTHrP(1–34) reduced a raised baseline in bladder smooth muscle preparations that had been precontracted with CCh (0.1 μM) (Figure 5, A and B). However, the inhibition of CCh-induced contraction was relatively mild compared with the prominent effect observed with spontaneous contraction. Other parameters, peak amplitude, interval time, and area under curve, were not changed significantly (Figure 5, C–E). Thus, inhibition of spontaneous contraction was considered to be a more relevant physiologic function of PTHrP in bladder smooth muscle than inhibition of contraction triggered by cholinergic stimulus.

Effect of PTHrP on cystometrogram

Finally, we performed a rat cystometrogram to investigate the in vivo effect of PTHrP. Intravenous administration of 10 μg PTHrP(1-34) rapidly induced a significant but transient increase in bladder compliance and a decrease in maximum intravesical pressure (Figure 6, A, B, and D). The treatment did not affect the voiding interval (Figure 6C) and residual urine volume. These findings demonstrate that PTHrP is a physiologically relevant bladder relaxant in vivo.

Discussion

This report demonstrates that PTHrP is up-regulated in the bladder under distension, inhibits spontaneous contraction of detrusor smooth muscle expressing PTH1R, and alters compliance and voiding contraction amplitude of the bladder. These findings suggest physiologic significance of the PTHrP/PTH1R axis in regulation of bladder function.

Bladder distension resulting from acute or chronic difficulty to void is a common urologic phenomenon. Mechanical stretch on smooth muscle (7, 29), as well as hypoxia with hypoperfusion (4, 30) and other stressors, can cause transcriptional changes that ultimately lead to myogenic alterations in bladder function (5). We performed comprehensive gene expression analysis in an in vivo bladder distention model. This model has potential advantages over the in vitro system because it reflects all direct and indirect factors affecting transcriptional changes in the

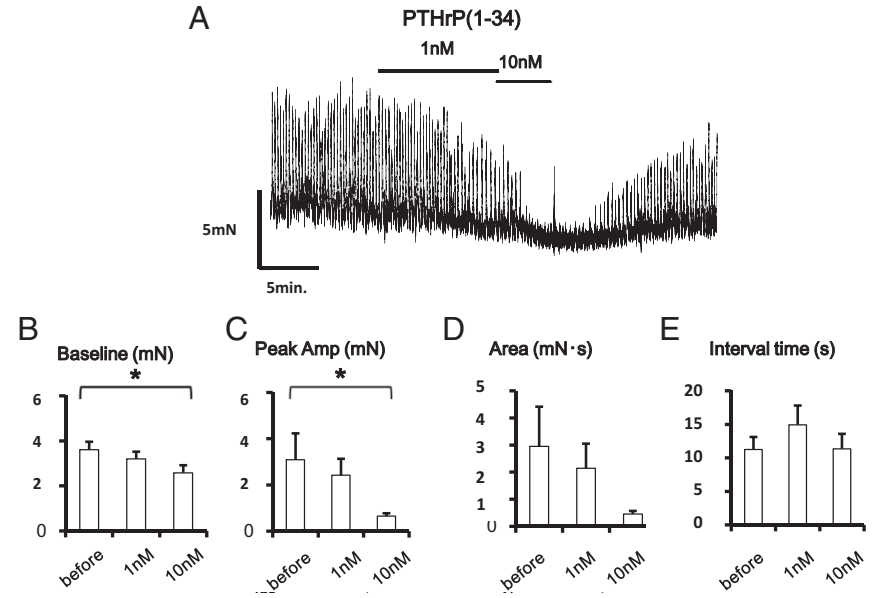

Figure 4. The Effect of PTHrP(1-34) on Cch-Induced Contractions of Rat Bladder Muscle Strips PTHrP(1-34) induced a moderate decrease in baseline tension and a marked decrease in peak amplitude (Amp) in rat bladder detrusor muscle strips without significant change of the interval time and area under curve, in a dose-dependent manner. $*P < .05$.

bladder, ie, muscle stretch, altered innervation, hypoxia, and hypoperfusion. In this model we rediscovered PTHrP, which has been reported in the literature as a stretch-induced gene in BSMC, as one of the most up-regulated genes by bladder distention (12, 13). Although a previous BSMC microarray did not identify PTHrP as a stretch-related gene (7), our review of the raw data of that microarray revealed that stretch did increase PTHrP, but one outlier datum among the 3 samples made the result statistically insignificant. In comprehensive analysis such as microarray important candidate molecules may be missed because of outlier data. Thus, multiple-array analyses on multiple models, like BSMC in vitro and bladder tissue in vivo, not only confirm the similarity of the two systems, but also provide a chance to pick up important candidate molecules missed by a single model. The other possibility is that, in addition to sheer stress itself, ischemia with bladder distension also up-regulated PTHrP (31, 32), and that an in vivo system generated a more distinct change in PTHrP expression than an in vitro stretch system without hypoxia.

An important finding of this paper is that PTH1R, the receptor of PTHrP, is predominantly localized in detrusor smooth muscle. This result and concurrent expression of PTHrP in detrusor smooth muscle indicate that PTHrP may function in an autocrine or paracrine fashion in smooth muscle. Although a recent study using immunohistochemistry reported that PTHrP was up-regulated in the urothelial layer of distended rabbit bladders (14), our results clearly show that the target tissue for PTHrP is not the urothelium because it expresses very low levels of PTH1R, and bladder PTH1R level remains constant under distension. Also, in the previous report, PTHrP up-regulation in the urothelium was not confirmed by biochemical methods like RT-PCR and immunoblotting, so that it could not be determined whether the increase in immunostaining signal was specific to PTHrP.

According to our localization study and the results from previous papers, we postulated that the PTHrP-PTH1R pathway may be a detrusor smooth muscle relaxant in the bladder. PTHrP acts via the AC-cAMP-PKA pathway in smooth muscle of many organs including blood vessels, gall bladder, and trachea (10, 24–26), and we confirmed that exogenous PTHrP(1–34) func-

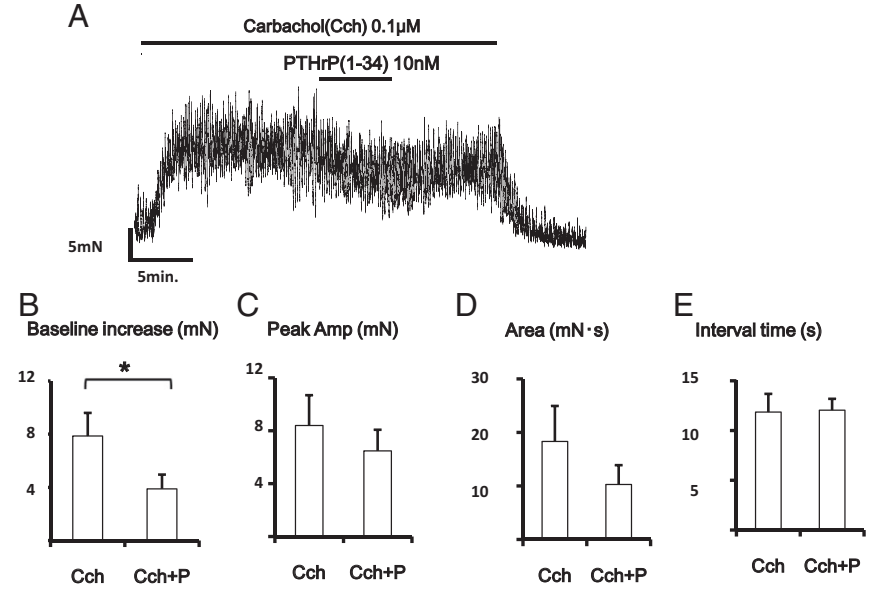

Figure 5. The Effect of PTHrP(1-34) on Cch-Induced Contractions of Rat Bladder Muscle Strips PTHrP(1-34) reduced the baseline increase of Cch-induced contractions in rat bladder detrusor muscle strips but had no significant effect on other parameters, including peak amplitude (Amp), area under curve, and interval time. Cch, carbachol treatment only; Cch+P, carbachol with PTHrP(1-34) treatment. $*P < .05$.

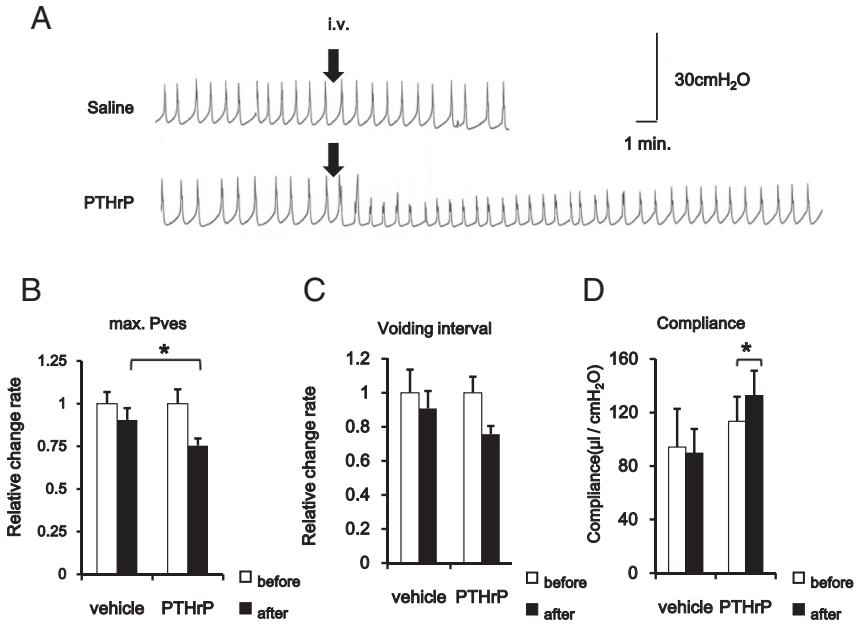

Figure 6. Cystometrogram Showing the in Vivo Effect of iv PTHrP(1-34) Administration A, Cystometrogram around iv administration of saline or 10μg PTHrP(1-34) amide. A typical trace of a rat is shown. B, PTHrP(1-34) decreased maximum intravesical pressure (max. Pves). The graph shows the relative change after 10 μg PTHrP(1-34) administration (n = 7-10). C, PTHrP(1-34) did not affect the voiding interval. The graph shows the relative change after 10 μg PTHrP administration (n = 7-10; $P = .092$). D, PTHrP(1-34) increased bladder compliance. Open bars show the compliance before administration, solid bars the compliance after administration (n = 7-10; $P = .396$ [saline], $P = .030$ [PTPrP(1-34] treatment). Mean value of the data before administration set at 1 for (B) and (C). max. Pves, maximum intravesical pressure. *$P < .05$.

tions under the same mechanism in vitro. Consistently, 8-bromo-cAMP, a cAMP analog, and forskolin, an AC activator, have an inhibitory effect similar to that of PTHrP (Supplemental Figure 1, A–C). However, we could not confirm the involvement of AC-cAMP-PKA pathway by inhibition study with H89, a PKA inhibitor (Supplemental Figure 2A), and SQ22536, an AC inhibitor (Supplemental Figure 2B), because these inhibitors themselves caused smooth muscle relaxation. The relaxant effect of H89 could have been due to Rho-kinase inhibition as reported previously (33). Although a muscle relaxant effect of SQ22536 is not reported in the literature, the relaxant effect of SQ22536 was reproducible in our experimental system. In addition, SQ22536 had no significant effect on relaxation of pulmonary vessels induced by PTHrP(1–34) (34), and this result was interpreted to mean that the action of SQ22536 may have varied with AC isoforms, and that AC isoforms involved in tension modulation may not have been sensitive to SQ22536 (35). In short, although the AC-cAMP-PKA pathway is likely to be the downstream pathway of PTH1R, it is not a definitive conclusion.

The most striking finding of this study is that PTHrP(1-34) potently suppresses spontaneous bladder smooth muscle contraction, while exerting only a modest inhibitory effect on Cch-induced bladder contraction as previously reported (12, 13). Spontaneous bladder contraction has been shown to be a very localized, asynchronous event in normal rat bladders (36, 37), but it becomes more synchronized with a larger amplitude after spinal cord transection (37) and bladder outlet obstruction (38). Such spontaneous contraction evokes afferent nerve firing in mouse bladders with detrusor overactivity (39) and may underlie abnormal increases in intravesical pressure (40). The sensitization of afferent fibers and/or increase in the magnitude of spontaneous contraction has been implicated in a number of bladder pathologies (41). Various inhibitory agents of spontaneous contraction have been reported to target β-adrenoceptor, Rho-kinase, intracellular calcium, and cAMP (42–45). Stretch-activated potassium channels may act as an intrinsic mechanism for suppressing bladder smooth muscle excitability (46). However, to our knowledge, an endogenous inhibitory ligand/receptor pair has not yet been established for spontaneous contraction, making our findings unique in the literature.

Another intriguing finding in this study is the effect of PTHrP(1–34) on cystometrogram change. It caused a rapid and temporal increase in bladder compliance and a decrease in maximum intravesical pressure. Although spontaneous contraction does not contribute to a phasic rise in the intravesical pressure of normal bladders (36), suppression of spontaneous contraction could be associated with increased bladder compliance. However, we should be cautious in interpreting all the observed cystometrogram changes as myogenic, because systemically administered PTHrP might work on other sites of the body, including nerves and sphincters. Thus, further studies may be needed to define precise links between PTHrP-mediated inhibition of spontaneous bladder contraction and in vivo bladder phenomenology.

Muscle remodeling in acute or chronic bladder outlet obstruction is often associated with detrusor overactivity, with increased muscle excitability and contractility (2, 4). PTHrP may counterbalance and alleviate the increased muscle contractility to avoid cell injury caused by excessive stretch or metabolic imbalance. Therefore, activation

of the PTHrP/PTH1R axis could be postulated as a therapeutic measure for treating pathologic conditions such as detrusor overactivity or low compliance bladders, the detrimental consequences of bladder outlet obstruction. Although PTHrP did not change the voiding interval and only subtly increased bladder compliance in healthy animals in our study, it could be expected that PTHrP, or its bladder-specific agonist, could be a therapeutic agent for pathologic conditions associated with detrusor overactivity, which should be investigated in further studies.

In conclusion, PTHrP acts as a unique endogenous relaxant of detrusor smooth muscle, preferentially inhibiting spontaneous contraction while suppressing cholinergic contraction to a lesser extent, and thus the PTHrP-PTH1R system may be a novel target for treating detrusor overactivity.

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